Acknowledgments

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Influence of Lauroyl and Myristoyl Peroxides and Oxidized Cottonseed Oil on Depot Fat and Liver Lipid Composition[1,2]

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In view of the interest in the biological properties of products of fat oxidation, lauroyl and myristoyl peroxides were fed and their nutritional effects compared with those of autoxidized cottonseed oil, which had been analyzed for its composition. Purified diets containing no fat + 2% of linoleic acid, 5% lauroyl or myristol peroxide, or 10% oxidized cottonseed oil were fed to weanling male albino rats for 73 to 98 days, after which they were killed and their organs weighed. Their sera, livers, and testicular fat bodies were used for lipid analysis.

With peroxides, growth was significantly depressed but not as much as when oxidized cottonseed oil was fed. Analysis of organ weight data showed that peroxides and oxidized cottonseed oil differed in their effects. Animals fed the latter had significantly heavier livers, kidneys, and hearts. The rats fed peroxides were also different from those fed the fat-free diet and those kept on restricted food intake. Gas chromatographic analysis of the testicular fat bodies revealed a greater deposition of oleate in the animals fed oxidized cottonseed oil, which suggested that these animals were unable to use the oxidized oil for depot fat formation. In the animals fed lauroyl and myristoyl peroxides, appreciable amounts of laurate and myristate, respectively, were found. The composition of the liver neutral fat of the animals fed peroxides was similar to that of the animals fed the low-fat diet + 2% linoleic acid. Serum cholesterol levels of the rats fed peroxides were about 70 mg. %, and of those fed oxidized cottonseed oil, 53 mg. %. The groups fed peroxides also had significantly higher liver cholesterol levels, which suggests that peroxides and oxidized cottonseed oil differed in their effects on cholesterol formation and transport.

THE NUTRITIONAL EFFECTS of substances developed in fats under the influence of heat and oxygen have been studied during the last decade. It has been pointed out that many of these materials are toxic (1,10,12). Later studies showed that oxidized fats or some of their fractions have other pharmacological properties, some of which seemed to invite further work (8,9,4).

Hardly any pharmacological studies of the materials formed in heated and aerated fats have been carried out because none of the polymers have been purified, and pure peroxides are not easily obtainable in sufficient quantities for nutritional work. In one such study, oleic peroxide was found not to influence growth (11). For the experiments reported, lauroyl and myristoyl peroxides were selected although they do not occur during autoxidation of fats. They are oxidation products of fatty acids and are readily available and relatively stable. Furthermore it seemed to us that biological data obtained with any products of fatty acid oxidation would be valuable. Their nutritional effects on rats were compared with those of a low-fat diet and one containing highly-autoxidized cottonseed oil in order to have some comparison of short-chain oxidation products and fat polymers. The animals were observed for body weight, organ weights, serum cholesterol levels, and composition of the neutral fat of the liver and testicular fat bodies.

TABLE I

Composition of Oxidized Cottonseed Oil

	%
Unsaponifiables	1.4
Fatty acids	51.5
Polymerized material	26.4
Carbonyls	9.4
Unknown	3.4
H_2O-soluble fragments	7.9

Fatty Acid Composition Based on Total Oil

	%
14 : 0 myristic	0.7
16 : 0 palmitic	20.4
16 : 1 palmitoleic	0.5
18 : 0 stearic	2.3
18 : 1 oleic	12.7
18 : 2 linoleic	14.5
20 : 0 arachidic	0.4
	51.5

[1] Aided by Grant A-1654 from the United States Public Health Service.
[2] Presented at the 34th fall meeting of the American Oil Chemists' Society.

TABLE II

Composition of the Low-Fat Diet

	%
Alcohol-washed casein (N.B.C.)	30
Dextrose (Cerelose)	64
Salt mixture (USP XIII)	3.5
Calcium carbonate	0.5
Cellulose (Alphacel)	2
	mg./kg.
Vitamins	
Choline dihydrogen citrate	1000
Inositol	1000
p-Aminobenzoic acid	300
Nicotinamide	100
Vitamin K (Synkayvite)	10
Thiamine hydrochloride	2
Pyridoxine hydrochloride	4
Riboflavin	4
Calcium pantothenate	10
Folic acid	2.5
Biotin	0.025
Vitamin B_{12} (.1% trituration)	5
Ascorbic acid	25
alpha-Tocopherol acetate	100
Free *alpha*-tocopherol	20
beta-Carotene	10
Vitamin D_2	0.5

Materials and Methods

For lauroyl peroxide [3] and myristoyl peroxide [4] we had to settle on concentrates which were at least 95% pure and had such slight odors that it is unlikely that the diets were unpalatable for this reason.

The oxidized cottonseed oil was prepared by aerating refined cottonseed oil at 95°C. for 240 hrs. The oxidized oil was analyzed for unsaponifiables, polymerized material, carbonyls, fatty acids, and volatile plus water-soluble cleavage products. The data are shown in Table I. The unsaponifiables were determined by a modification of the Kerr-Sorber method on a separate portion of the oil. The other constituents were determined by gas-liquid chromatography (GLC) on a succinate polyester column in conjunction with the following procedures, which admittedly have not been thoroughly evaluated but which seem reasonable and should give a close approximation.

A portion of oxidized oil, to which a known amount of methyl pentadecanoate had been added as an internal standard, was converted to methyl esters by methanolysis with potassium methylate and the esters were recovered. For determining polymerized material, chromatograms (GLC) were obtained from a small amount of these esters and from the same amount [5] of known pure fatty acid methyl esters for comparison under identical conditions. Since peaks are not expected from polymers, the difference in total area of the peaks on the chromatograms, when related to the peak area of the internal standard and its concentration in the oxidized oil, represent an estimate of the nonvolatile polymerized material. Similarly a comparison of the peak area on the chromatograms of methyl esters from the oxidized oil before and after removal of carbonyls (again related to the internal standard) represents a measure of carbonyl material in the sample.

To remove carbonyls a 15- to 20-mg. portion of methyl esters of oxidized oil was placed in a test tube with approximately 75 mg. of 2,4-dinitrophenylhydrazine. Five ml. of methanol were added, and the mixture was brought to a boil. The heat was removed, and 0.2 ml. of concentrated HCl was added. The mixture was then boiled for an additional 5 min. Most of the methanol was removed by blowing a stream of nitrogen over the mixture. The residue was extracted with petroleum ether, and the extracts were added to a column 1 cm. I.D., containing 3 g. of 80% silicic acid–20% filter aid. The carbonyl-free esters were eluted with 150–200 ml. of petroleum ether.

Some error may be incurred at this point since the peaks were selected as weight percentage, and this may not be entirely true when methyl esters of fatty acids and carbonyls are both present in the sample. The individual fatty acids were calculated from the chromatogram of the carbonyl-free sample from the ratio of their peak area to the peak area of the internal standard. Several broad unidentified peaks, which were neither normal esters nor carbonyls, appeared on the chromatogram. These were calculated as unknown contituents. After all of the aforementioned constituents were calculated to the original oil, the portion of sample unaccounted for was presumed to be water-soluble and volatile material lost in the recovery of the esters. This value would probably be the least accurate since it was determined by difference and would reflect the sum of all errors in the procedure.

Three separate series of experiments were carried out on weanling male albino rats from a homogeneous colony. The animals were ear-marked and weighed at weaning and reweighed 5–15 days later, at which time they were distributed into matching groups so that the average weights of the groups were equal at weaning and again at the subsequent weighing. This made it probable that the groups in any one series had similar growth tendencies. After the groups had been made up, they were transferred to the experimental diets. At the end of the experimental periods the animals were killed, their organs weighed, and their sera, livers, and testicular fat bodies saved for cholesterol determinations and gas-liquid chromatography.

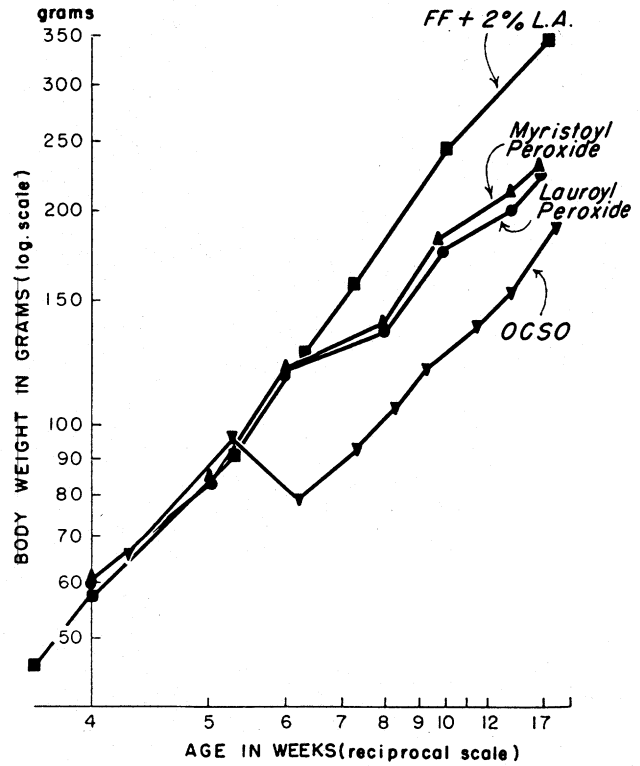

FIG. 1. Growth of rats fed a low-fat diet supplemented 2% linoleic acid or one containing lauroyl or myristoyl peroxide or autoxidized cottonseed oil.

[3] *Alperox C*, Lucidol Division of Wallace and Tiernan, Buffalo, N.Y.
[4] Lucidol Division, Wallace and Tiernan, Buffalo, N.Y.
[5] It had been shown that the peak areas could be reproduced to within ± 2.5% by a carefully standardized technique, employing a micro syringe in which the plunger extended to the tip of the needle.

For the feeding studies, highly purified diets were used. Table II gives the composition of the low-fat diet fed with a 2% linoleic acid supplement, to the controls for 98 days. Modifications of this diet for the other experimental groups are given below.

The groups fed peroxides were given one of these materials mixed with 3 to 4 g. of a diet similar to that in Table II but without vitamin or linoleic acid supplements. This was prepared fresh every day to prevent destruction of the peroxides. Linoleic acid and vitamins were omitted to avoid reactions between them and the peroxides. As soon as the rats had eaten their ration, they were given free access for two hours to the low-fat diet with supplements. This permitted a sufficient linoleic acid and vitamin intake. Food was then withdrawn until the next morning's feeding because they ate the peroxides only when they were hungry. This procedure was carried out five days a week. From Friday afternoon until Sunday noon they were given the supplemented diet *ad lib.*, after which food was withdrawn. In the early part of the experiment they were given 0.6 g. of peroxides + 3.4 g. of diet as their daily ration. This was gradually changed to 1.0 g. of peroxide + 3 g. of diet per day. The total intake of peroxides for the experimental period was 36 g. for each rat. After 72 days on the diet the animals were permitted to eat freely of the supplemented low-fat diet for 36 hrs. and were then weighed and killed.

The oxidized cottonseed oil (OCSO) diet was prepared by substituting 10% of the oil for 10% of the dextrose in the diet shown in Table II (omitting linoleic acid). The group was killed after 95 days on the diet.

For the gas chromatographic analyses, pooled samples of liver and depot fat were homogenized while frozen and extracted with 3:1 ethanol:ethyl ether and filtered. The solvent was evaporated, and the extract was redissolved in petroleum ether, which was then evaporated under vacuum. The extract was saponified with alcoholic KOH, washed with ethylene dichloride, acidified, extracted with ether, and dried. This material was hydrolyzed with HCl in 2:1 methanol:benzene; the solvent was evaporated off and the residue

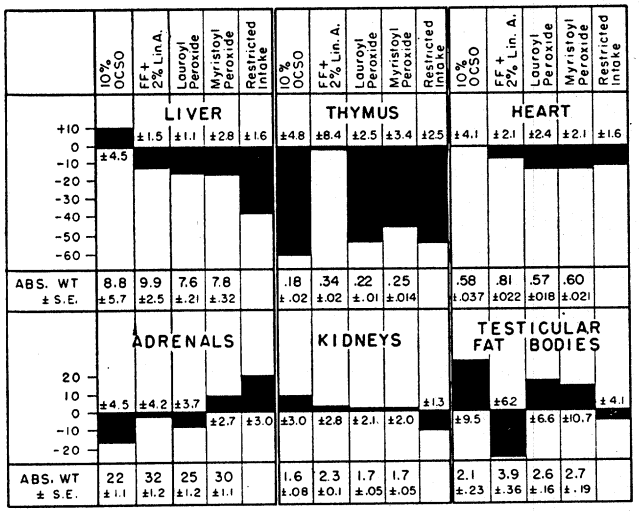

FIG. 2. Absolute weights and their percentages of deviation from normal of organs of rats fed a low-fat diet suupplemented with 2% of linoleic acid or diets containing lauroyl or myristoyl peroxide, 10% of autoxidized cottonseed oil, or 10% of fresh lard. The latter group were given only enough diet to enable them to maintain their weight.

TABLE III

Percentage Composition of the Neutral Fat of the Testicular Fat Bodies and Livers of Rats Fed a Low-Fat Diet + 2% of Linoleic Acid or One Containing Lauroyl Peroxide, Myristoyl Peroxide, or Autoxidized Cottonseed Oil

Fatty acid	Testicular Fat Body Fat				Liver Fat		
	Fat-free + 2% lin. acid	Lauroyl Peroxide	Myristoyl Peroxide	Ox. cottonseed oil	Fat-free + 2% lin. acid	Lauroyl Peroxide	Myristoyl Peroxide
Caprylic	.1	.1	.1	.02	....	....	....
Capric	....	....	....	.03	....	.1	....
Lauric	....	6.0	.1	.04	....	.1	....
—2H	....	.1	....	....	....	....	....
Myristic	1.5	3.1	8.4	.6	.3	.5	.2
—2H	.5	.2	.6	.2	....	....	....
Palmitic	28.4	28.2	26.4	27.4	32.0	36.7	33.4
—2H	17.5	14.8	14.6	17.2	....	....	....
Stearic	.5	.7	1.3	.5	18.4	20.8	17.6
Oleic	30.4	37.7	39.7	47.7	17.3	18.7	25.1
Linoleic	21.1	9.1	8.8	5.3	13.2	6.5	7.6
Arachidonic	....	....	....	....	16.1	15.1	13.2

dissolved in ether, which was then washed with water and 10% Na_2CO_3. The ether extract was dried over anhydrous Na_2SO_4 and evaporated under N_2. The methyl esters were diluted with chloroform, and their fractograms were obtained on a Perkin-Elmer Vapor Fractometer using a 2-meter polydiethylene glycol succinate column at 220°C.

The cholesterol determinations were carried out according to Sperry and Webb (13).

Experiments

Figure 1 gives the average growth curves of the groups fed the low-fat diet + 2% of linoleic acid, myristoyl peroxide, lauroyl peroxide, and 10% OCSO. Body weights are plotted on a logarithmic scale against the reciprocal value of the age. When plotted in this way, the weights of normally growing animals lie on a straight line of known slope (14).

Growth of the animals fed peroxides was significantly less than that of the animals maintained on the low-fat diet + 2% of linoleic acid, the growth of which had the predicted slope. The group fed OCSO lost weight after being transferred to the diet, then gained weight; however they were unable to attain the weight of normal animals. It is not certain whether the significant difference in the body weights of this latter group and those fed peroxides was due to a difference in toxicity or to the difference in levels at which the materials were included in the diet. The animals given myristoyl peroxide were somewhat heavier than those fed lauroyl peroxide, but the difference was not significant. We had the impression that myristoyl peroxide was eaten more readily than lauroyl peroxide.

Direct comparison of organ weights of the various groups was difficult because of differences in their average body weights. To overcome this difficulty, the organ weights were compared with those of normal animals of the same body weights which had been fed a similar diet containing 10% fresh fat. Data from over 400 male rats were used to obtain the standard organ weight–body weight distributions, the average points of which lie on straight lines when plotted on log-log paper. From these lines the expected weight of an organ at any body weight could be read. The differences between the expected weights and the actual organ weights of the experimental animals were expressed as percentages of the expected weights and averaged for each group. These "percentages of deviation from normal" were used for comparisons between groups. Details of this method have been published elsewhere (5).

In Figure 2 are given the average organ weights and their percentages of deviation from normal. As in previous studies, the animals fed the low-fat diet + 2% of linoleic acid had adrenals, kidneys, and thymuses which were normal in weight and livers, hearts, and testicular fat bodies which were below normal (6). Also in agreement with previous observations, the animals fed OCSO had enlarged livers and kidneys, small thymuses, and normal hearts (5). They differed from previous groups in having heavier-than-normal fat bodies and smaller-than-normal adrenals. The latter may have resulted from the fact that the rats in this experiment were kept on the diet much longer than in earlier ones.

In the animals fed peroxides, livers, kidneys, and hearts were similar in weight to those of the animals fed the low-fat diet + 2% of linoleic acid, which also formed part of their own diet. As in the rats fed OCSO, thymus weights were markedly depressed, but there was a significant difference in the amounts of deviation of the groups fed OCSO and myristoyl peroxide (P less than .02). Their testicular fat bodies were heavier than normal. Thus the diets containing peroxides produced an organ weight pattern of their own. In fact, lauroyl and myristoyl peroxides differed from each other with respect to their effects on adrenal weights (P less than .01).

Because the food intakes of the groups fed peroxides were rather low, Figure 2 includes data from animals which had been kept on a restricted intake of a diet similar to that in Table II but containing 10% lard (6). When one compares the organ weight data from these animals with those from the peroxide-fed groups, distinct differences are apparent with respect to adrenals, kidneys, and fat bodies. Therefore reduced food intake cannot be the whole explanation of the organ weight changes seen in the groups fed peroxides.

Table III gives the composition of the neutral fat in the testicular fat bodies. The fat bodies of the animals fed the fat-free and OCSO diets contained only small amounts of acids shorter than C_{16} whereas feeding of lauroyl and myristoyl peroxides led to the deposition of considerable amounts of laurate and myristate, respectively. All groups were the same with regard to the deposition of C_{16} and stearic acids. A higher level of linoleate occurred in the group fed the low-fat diet + 2% of linoleic acid than in any other group. Oleate deposition was much greater with OCSO than with the other diets and was equally high in four other groups fed 10% OCSO (not included in the table). The composition of the depot fat of the animals fed OCSO resembled, except for its linoleate content, the fat of animals fed a low-fat diet with little or no added linoleic acid, especially with regard to the high oleate content of the depot fat (2). However, inasmuch as the fat bodies of the rats fed OCSO contained an appreciable amount of linoleate, linoleic acid deficiency could not have been the cause of these findings. In view of the fact that feeding of fresh fats in the presence of sufficient linoleate results in lower oleate levels in the depot fat than in animals fed a low-fat diet (7), it is probable that the high oleate content of the depot fat in the rats fed OCSO indicates that the animals had difficulty in using OCSO for depot fat formation.

Results of the liver neutral fat analyses are included in Table III. As was to be expected (3), the liver fat contained more of the highly unsaturated acids, such as arachidonic acid, which was not found in the depot fat. Moreover the liver fat contained appreciable amounts of stearate and less of oleate. The composition of the liver fat of the animals fed peroxides did not differ from that of the group fed the low-fat diet except that the linoleate level of the former was half that of the group fed the low-fat diet.

TABLE IV

Liver Lipid and Liver and Serum Cholesterol Levels of Rats Fed Lauroyl or Myristoyl Peroxide or Autoxidized Cottonseed Oil

	Serum cholesterol (mg. %)	Total liver lipids (% dry wt.)	Liver cholesterol (mg. dry wt.)
Lauroyl peroxide	70 ± 2.1	21.0 ± 1.0	915 ± 33
Myristoyl peroxide	71 ± 3.0	22.4 ± 1.05	853 ± 53
Ox. cottonseed oil	53 ± 2.4	21.6 ± 0.6	768 ± 14

Table IV gives results of other lipid analyses. The serum cholesterol levels of the animals fed peroxides were approximately those previously seen in animals fed 8% lard or a low-fat diet + 2% of linoleic acid. These values were significantly higher than those of rats fed oxidized cottonseed oil. The total liver lipids were about the same in animals fed peroxides and OCSO and were similar to those found in rats fed 8% fresh lard (9). The liver cholesterol values of the animals fed peroxides were significantly higher than those of the rats given OCSO. The circumstance that both serum and liver cholesterol levels were significantly lower in the animals fed OCSO than in those fed peroxides may suggest that cholesterol synthesis in the animals fed OCSO was depressed.

The data show that the metabolic effects of peroxides and oxidized cottonseed oil differed from each other sufficiently to conclude that they were not merely an expression of quantitative differences in the amounts of the materials fed. Oxidized cottonseed oil is, of course, a conglomerate of substances, but one of its most active components is its polymer fraction, which probably accounts for the difference observed.

This study is a small step in the direction of investigating the effects of more purified oxidation products of fatty acids. In the light of these and previous experiments (8,9) one may hope that purification of the wealth of substances occurring in oxidized fats may yield important biological tools.

Acknowledgments

We are grateful for the continued advice and criticism of Waldo Ault, Daniel Swern, and H.B. Knight of the Eastern Regional Research Laboratory, and to Robert Kross of the Food and Drug Research Laboratory for his work. We wish to thank the Lucidol Division, Wallace and Tiernan Inc., Buffalo, N.Y., for kindly supplying the myristol and lauroyl peroxides, and Leo Pirk, Hoffmann-La Roche Inc., Nutley, N.J., for the vitamins used in these studies.

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Report of the Smalley Committee, 1960-61

SEVEN SUBCOMMITTEES distributed nine different series of samples this season. Table I lists the types of samples and the distribution of participation by 514 collaborators on the analysis of 4,541 samples. In addition, a number of samples were distributed free of charge to inquiring laboratories to encourage future participation by new collaborators. Subscription to the program this year showed an increase of about 1.5% over last season.

TABLE I

Type of sample	Number of collaborators	Number of samples	Determinations per sample
Cottonseed	48	10	6
Soybean	37	10	2
Peanut	10	7	5
Meal	142	15	3–4
Vegetable oil	81	6	3
Tallow and grease	91	5	7
Glycerine	26	5	3–5
Drying oil	16	6	5
Edible fat	63	5	14

Each subcommittee has furnished its collaborators with a final report, summarizing the work and listing relative standings.

As of April 10 the Smalley account showed the following: receipts, $7,570.47; expenses, $6,675.49; and net $894.98. The balance may be decreased by as much as $100 by a few outstanding expenses. However it may be conservatively estimated that our total expenses will be about $200 less than last year while our total receipts increased by $321.47. Unless postal rates are increased considerably we should not have to increase our prices. A detailed account has been given to the Governing Board.

The subcommittee chairmen have expressed their thanks to all of those to whom we are indebted for making the Smalley program a success this year. To acknowledge them individually would be impractical in this report, but we are nonetheless appreciative of their contributions.

The previously-established grading systems were used again this year. Although they are reasonably sound, we hope to improve some weak spots and, in some cases, to tighten tolerances in line with the increased excellence of analytical work.

A new award will be presented this year in the form of a trophy given by the personnel of the Barrow-Agee Laboratories to honor the memory of E.R. Barrow and G.W. Agee. This award will be given each year to the collaborator attaining the highest grade in the cottonseed series. The Barrow-Agee Cottonseed Trophy will be permanently retired by a collaborator who wins it three times. The first leg on this trophy has been won by Paul D. Cretien of the Texas Testing Laboratories, Dallas, with a grade of 99.70%.

Other Smalley certificates of proficiency to be presented this year are as follows.

Cottonseed. With 48 chemists participating, second place was attained by J.R. Mays Jr., Barrow-Agee Laboratories, Memphis, Tenn., with a grade of 98.80.

Soybean. Of the 37 chemists participating, five of them tied for first place with perfect scores. Certificates will be given to D.A. Bradham Jr., Barrow-Agee Laboratories, Greenville, Miss.; W.N. Kesler, Woodson-Tenent Laboratories, Little Rock, Ark.; J.G. Bowling, Woodson-Tenent Laboratories, Des Moines, Ia.; W.G. Wadlington, Woodson-Tenent Laboratories, Chicago, Ill.; and Nick Brokamp, Archer-Daniels-Midland Company, Cincinnati, O.

Peanut. First place among 10 chemists was won by T.C. Law, Law and Company, Atlanta, Ga., with a grade of 98.80. Second went to Philip C. Whittier, Law and Company, Montgomery, Ala., with 98.64.

Tallow and Grease. Participating chemists numbered 91. First place was given to A. Dennis Caeton, Los Angeles Soap Company, with a grade of 100; second was given to F.A. Adams, Procter and Gamble Company, Long Beach, Calif., with 99.36.

Edible Fat. With 63 chemists participating, two tied for first place with grades of 99.44: M.W. Felker, Anderson, Clayton and Company, Sherman, Tex.; and William Stewart, Swift and Company, Atlanta, Ga.

Drying Oils. Sixteen chemists participated; first place was won by O.W. Johanson, Archer-Daniels-Midland Company, Minneapolis, Minn., with 94.50; and second, by V.F. Bloomquist, Minnesota Linseed Oil Company, Minneapolis, with 94.25.

Glycerine. Of the 26 chemists who participated, four tied for first place with perfect scores: T.S. McDonald, Procter and Gamble Company, Dallas, Tex.; A.H. York, Procter and Gamble Company, Cincinnati, O.; J.H. Dietz, Harshaw Chemical Company, Gloucester City, N.J.; and F.D. Newcomb, Lever Brothers Company, Los Angeles, Calif.

Vegetable Oils. First place among 81 chemists was won by W.J. Howard, HumKo Company, Champaign, Ill., with a perfect score. Ben C. White, Barrow-Agee Laboratories, Shreveport, La., and F.M. Tindall, HumKo Company, Memphis, Tenn, were tied for second place with grades of 99.4. After a recalculating of the results, with no tolerances allowed, the ties remained unchanged. Both were given certificates.

Meal. This is the original Smalley series and continues to have the largest participation. There were 142 chemists this year. Samples were sent to Canada, Mexico, and South America.

First place for moisture went to Biffle Owen, Plant-